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Progression of cancer cells from a non-invasive, non-metastatic phenotype to an invasive and metastatic phenotype is associated with the expression of a variety of cell surface proteases. Primary among these are the zinc metalloproteases known as matrixins, which include matrix metalloproteases such as collagenases and stromelysins. Matrixins can degrade extracellular matrix proteins, contributing to metastasis and angiogenesis. Another family of zinc metalloproteases are the 'astacins', which include the extracellular proteases meprin A and B. The expression of a novel form of meprin β mRNA only in cancer cells, and the secretion of meprin A protein by colon carcinomas has been previously documented. This indicates that these proteases play a role in cancer cell progression. The current project focuses on the expression of meprins A and B in breast cancer cells, and their potential role in tumorigenesis, invasion and metastasis.

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Jack Matthews 5/30/00

PI - Signature Date

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Introduction

The objectives of year 2 of award number DAMD17-98-1-8143 were to express the extracellular metalloproteases meprin α and β in a cultured human breast cancer cell line. A suggested role for meprins in cancer cells is based on observations of meprin expression in several types of cancer cells, especially colon cancer, and on the in vitro proteolytic activity of these enzymes. We have shown that meprin β mRNA is present in many types of cultured human cancer cell lines, including breast, colon and pancreas (Matters and Bond, 1999). Meprin α protein is secreted from a metastatic colon cancer cell line (SW620) but not from a nonmetastatic colon cancer cell line (SW480) (Matters and Bond, unpublished data). Others have reported that human meprin α is secreted from Caco-2 colon cancer cells and from colorectal tumors (Lottaz et al., 1999). In vitro, meprin α and β can degrade ECM components as well as bioactive peptides, but each enzyme has unique substrate specificities (Bond and Beynon, 1995, Bertenshaw et al., unpublished data). Therefore, our hypothesis is that the proteolytic activities of meprins may contribute to tumor growth and/or to the metastatic potential of breast cancer cells.

To address this question, constitutively expressed meprin cDNA clones have been stably transfected into a moderately metastatic breast cancer cell line, MDA-MB-231. Once these stable transfectants are characterized, the human breast cancer cells overexpressing the meprin subunit proteins, and vector transfected controls, will be tested in vitro for their growth properties and invasiveness, and also injected in nude mice to determine their tumorigenicity and metastatic behavior (Objectives for years 3 and 4). This will assess the effect of meprin overexpression on the metastatic potential of breast cancer cells in in vitro and in vivo systems. These experiments will address whether overexpression of meprin protein affects tumor growth, or may convert a moderately metastatic breast cancer cell to a highly metastatic cell. Thus, the objectives of year 2 were to create and characterize stably transfected breast cancer cell lines which overexpress the meprin α and β proteins.

Body of the Report

At the end of year 1 of this award, full-length cDNA clones encoding the meprin α and β subunits in the constitutive mammalian expression vector pcDNA 3.1(+)(Invitrogen) had been made. The human meprin α cDNA was stably transfected into HEK 293 cells, a human kidney cell line routinely used for expressing meprin. Several clones expressing high levels of the protein were selected. The human meprin α cDNA was also transfected in the nonmetastatic human breast cancer cell line MCF7. In contrast, when the human meprin β cDNA was transfected into HEK 293 cells, no stable transfectants or transient transfectants expressing the human meprin β protein could be detected. This result is similar to the problems experienced when expressing the mouse meprin β cDNA in HEK 293 cells, where no expression of the mouse enzyme can be detected either. However, stable HEK 293 clones expressing the rat form of the meprin β protein were obtained and characterized. Thus, all further work on meprin β expression will be done using the rat meprin β cDNA. For consistency, a rat α meprin cDNA clone in the mammalian expression vector pcDNA 3.1(+) was also constructed. The rat meprin α cDNA produced many clones with high levels of meprin α protein expression in HEK 293 cells. Therefore, both meprin α and β cDNA clones that reliably produced recombinant protein in cultured cell lines were available.

At the beginning of year 2, the experimental focus was on characterizing the MCF7 breast cancer cell lines which had been transfected with a human meprin α cDNA. Initial screening of meprin α transfected MCF7 clones by RT-PCR revealed several clones which expressed low levels of the human meprin α mRNA. These clones were then examined for the expression of meprin α protein by Western blots. No meprin α protein expression was detected in any of the MCF7 clones which had showed meprin α mRNA expression. In comparison to transfected HEK 293 cells, the level of meprin α mRNA expression in the MCF7 transfectants was considerably lower, thus it appears that the level of meprin protein in the MCF7 clones may have been below the level of detection by Western blot. Alternatively, the meprin α protein may have been rapidly turned over by the MCF7 cells. Rather than repeat the unsuccessful MCF7 transfection, another human breast cancer cell line, MDA-MB-231, was chosen to continue this study.

The MDA-MB-231 breast cancer cell line, obtained from Dr. Dan Welch, Jake Gittlen Cancer Center, Penn State College of Medicine, is moderately metastatic in nude mice models and can be easily transfected using lipid-based transfection reagents. Therefore, this cell line is better for determining whether meprin can increase invasive or metastatic characteristics of breast cancer cells than the nonmetastatic MCF7 cell line

previously used. In addition, a more highly metastatic breast cancer cell line, MDA-MB-435, was also obtained from Dr. Welch. Both these breast cancer cell lines were screened for endogenous expression of the meprin α and β mRNAs by RT-PCR and for meprin protein by Western blot. Western blots of media and cell membrane fractions were probed with anti-meprin α and meprin β antibodies, respectively. No detectable meprin protein was found in either the MDA-MB-231 or MDA-MB-435 cell or media fractions. However, RT-PCR done with meprin β primers did detect a low level of transcript in both breast cancer cell lines. As with the MCF7 cells, the meprin protein may be at a level too low to detect or rapidly turned over. Another possible reason for the lack of detection of the endogenous human meprin protein may be the quality of the meprin antibodies currently available, none of which were generated against human proteins. Recombinant human meprin α protein has recently been purified (Han and Bond, unpublished), and we are using this protein to make a human meprin α polyclonal antibody. A polyclonal rat meprin β antibody is also being produced and should be available shortly.

Because the rat meprin α and β cDNAs gave consistent expression of meprin protein in HEK 293 cells (Year 1 of this award), these cDNAs were used to transfect MDA-MB-231 cells. Using Lipofectamine 2000 (Life Technologies), MDA-MB-231 cells were transfected with the rat meprin α and β cDNAs as well as with the vector plasmid only (pcDNA 3.1+) as a negative control. MDA-MB-231 clones expressing the meprin α or meprin β cDNAs were obtained. Clones expressing both high and low levels of meprin protein, based on Western blots (Fig. 1), were selected for further study. The availability of MDA-MB-231 cells expressing a range of meprin protein, from high levels to barely detectable on Western blots, will prove useful in in vivo studies. We will be able to compare MDA-MB-231 cells expressing different amounts of meprin for their tumorigenicity and metastatic potential in mice models.

MDA-MB-231 clones expressing high levels of meprin α or meprin β were fractionated into soluble and membrane bound-proteins, and the media containing secreted protein was also collected and concentrated. Membrane-bound protein was released by treatment with 1% octylglucoside, and the presence of meprin in the soluble, membrane, and media protein fractions were detected by Western blots (Objective 2, Task 1). In the mouse kidney and intestine, as in HEK 293 cells, the meprin α subunit protein is secreted from the cell if meprin β is not present, while the meprin β subunit protein stays anchored at the cell surface through a short transmembrane domain. Most of the meprin β subunit protein is extracellular, and through covalent and noncovalent α/β interactions, the meprin α protein can associate with meprin β subunit protein and be maintained at the cell membrane. Fractionation experiments showed that the transfected MDA-MB-231 cells also secrete the

meprin α subunit protein into the media and retain the meprin β subunit protein at the cell membrane. Because no meprin β protein is present on the meprin α transfected MDA-MB-231 cells, all the meprin α protein was secreted instead of being associated with the cell membrane. Vector transfected control cells showed no evidence of meprin protein on the cell membranes or in the media. Experiments are currently underway to make a double transfectant of MDA-MB-231 cells with both the meprin α and β. Expressing both meprin α and meprin β protein will test whether α/β cell surface oligomers will be formed in MDA-MB-231 cells as they are in other cultured cell systems.

Most extracellular proteolytic enzymes are regulated in part by their secretion in a latent or proenzyme form. Activation of proteases often involves the removal of a propeptide region by another type of proteolytic enzyme at or close to the cell surface. Both meprin proteins are secreted as proenzymes in recombinant expression systems, such as HEK 293 cells. However there is preliminary evidence that cancer cells, such as the SW620 colon cancer cell line, can activate meprin α. Therefore, it was important to determine if MDA-MB-231 cells could activate the meprin α or β proteins. Meprin proteins expressed in MDA-MB-231 cells were treated with a mild trypsin solution (25 ng/μl) for 30 minutes at room temperature. A shift in the size of the meprin protein, as detected by Western blots, indicates removal of the propeptide from the N-terminus. Both the meprin α and β proteins showed a decrease in size with trypsin treatment, indicative of proenzyme activation (Fig. 2). Thus, in MDA-MB-231 cells in culture, meprin α and β proteins are secreted as inactive forms. In addition, this also implies that the meprin proteins are folded properly and are stable. Previous work in our lab has demonstrated that misfolded meprin proteins, such as mutant proteins with truncations or deleted domains, are susceptible to complete degradation by trypsin (Tsukuba and Bond, 1998).

Another characteristic of meprins, as with most extracellular proteases, is a high degree of protein glycosylation. However, cancer cells can alter the glycosylation patterns of proteins. The deglycosylating enzymes EndoH, which removes high mannose type glycosylation, and EndoF, which removes all N-linked sugars, were used to analyze the type and degree of glycosylation on the meprin proteins. After overnight treatment with the deglycosylating enzymes, meprins were subjected to Western blotting. The meprin β protein expressed in MDA-MB-231 cells showed a pattern of deglycosylation identical to that of meprin β protein expressed in HEK 293 cells. No high mannose-type sugars were present on the protein, indicating that the meprin protein was complex glycosylated, and the size of the untreated and deglycosylated proteins was identical, indicating that the degree of meprin glycosylation in the different cells was similar.

Key Research Accomplishments

- Screening of MDA-MB-231 and MDA-MB-435 human breast cancer cell lines for endogenous expression of meprins.
- Production of stably transfected lines of MDA-MB-231 human breast cancer cells expressing either the meprin α or β protein.
- Characterization of the recombinant meprin proteins produced by MDA-MB-231 breast cancer cells.

Reportable Outcomes

1.) Manuscripts and Abstracts:

During year 2, I was a co-author on a paper entitled "Structure of the mouse metalloprotease meprin β gene (*Mep1b*): Alternative splicing in cancer cells" by W. Jiang, J. Kumar, G. Matters, and J. Bond. This manuscript, which characterizes the mouse meprin β gene (*Mep1b*) and describes how alternatively spliced exons used only in mouse cancer cells are arranged, is in press in the journal Gene. In April 2000, I was invited to attend the Gordon Research Conference entitled "Proteolytic Enzymes and their Inhibitors", to be held on July 9-13, 2000. I will be giving a poster presentation at that meeting.

I was a co-author on abstracts for posters presented at the two conferences by a student in the lab, Greg Bertenshaw. The first was at the conference entitled "Towards an Understanding of Tolloid Proteinases" at the University of Manchester, Manchester, England in May 1999, and the second was at the IPS (International Proteolysis Society) meeting on Mackinac Island, Michigan in September 1999. Copies of the abstracts for these posters is enclosed. I am also a co-author on the abstract for a talk entitled "Meprins-Metzincins with unique properties and expression patterns". This presentation will be made by my mentor, Dr. Judith Bond, at a conference entitled "International Symposium on Proteases: Basic Aspects and Clinical Relevance" in Montebello, Quebec, Canada in June 2000.

A portion of my work was described at research seminars given by Dr. Bond at the Roswell Park Cancer Institute in Buffalo, N.Y. (October, 1999) and at the Emory University School of Medicine, Atlanta, GA (April, 2000).

2.) Development of Cell Lines:

Two stably transfected clones of the human breast cancer cell line MDA-MB-231, one expressing the meprin α subunit protein and one expressing the meprin β subunit protein, were created. These cell lines will be crucial to upcoming in vivo studies, where

the effects of meprin overexpression on the tumorigenicity and metastatic behavior of breast cancer cells in a mouse model system will be tested.

3.) Opportunities:

Because of funding of award number DAMD17-98-1-8143, I applied for and, in April 2000, I was accepted as a member of the Penn State College of Medicine Cancer Center (see attached letter). The Penn State Cancer Center is in the process of applying for status as an accredited Cancer Center through the NCI. This procedure is in the planning stages, and I will be participating in activities related to the Penn State Cancer Center accreditation process. As a member of the Penn State Cancer Center, I will have opportunities to establish new collaborations with clinical and basic cancer researchers at this institution (The Hershey Medical Center) as well as at the main campus of Penn State University (University Park).

Conclusions

Based on the experiments done in year 2 of this award, the expression of recombinant meprin α and β proteins in the human breast cancer cell line MDA-MB-231 appears to be very similar to their expression in HEK 293 cells. The meprin proteins are expressed in the correct cellular compartment: on the cell membrane for meprin β and secreted into the media for meprin α. Both proteins are produced as trypsin-activable precursors and are glycosylated normally. This implies that these proteins are synthesized, folded and processed correctly in breast cancer cells. It is likely, therefore, that the meprin protein expressed in the breast cancer cells will function as wild-type meprin protein does. This will permit the remaining portions of the Objectives of this award to proceed as planned.

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Lottaz, D., Maurer, C.A., Hahn, D., Buchler, M.W., Sterchi, E.E. (1999) Cancer Research, 59: 1127-1133.

Matters, G., Bond, J. (1999) Mol. Carcin., 25: 169-178.

Tsukuba, T., Bond, J. (1998) J. Biol. Chem., 273: 35260-35267.

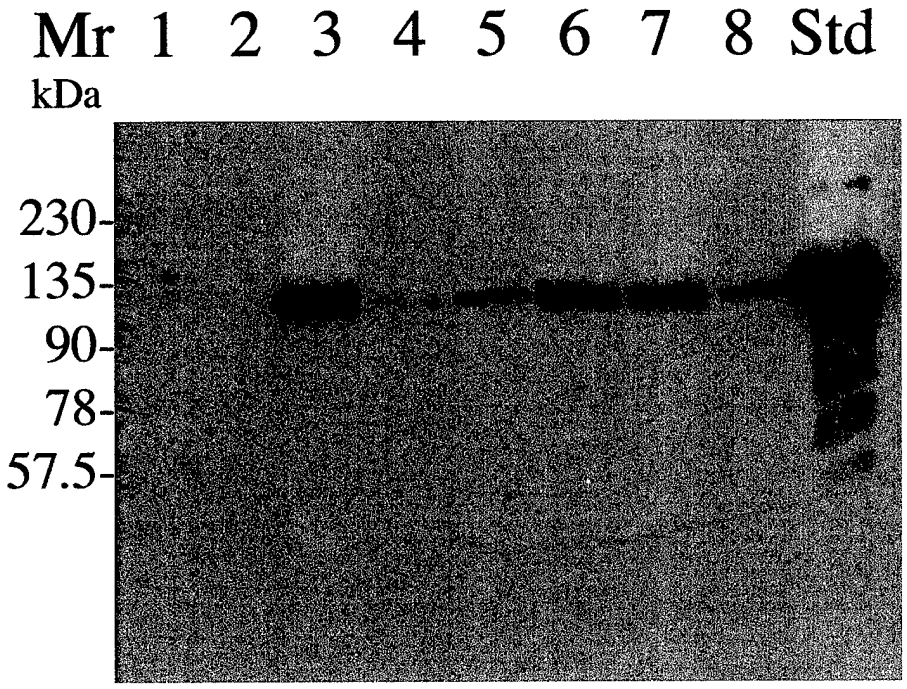
Appendices

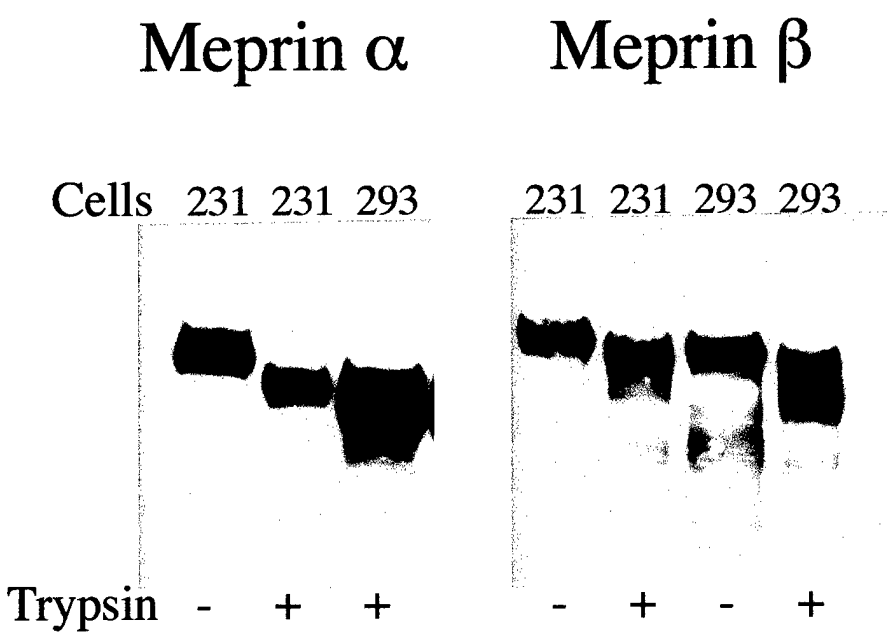
Figure Legends:

FIGURE 1. Western blot screen of MDA-MB-231 clones expressing the meprin β protein. Potential meprin β expressing cells were extracted by sonication, the membranes were fractionated by ultracentrifugation and resuspended in 1% octylglucoside in 20 mM Tris, pH 7.5. Membrane proteins were separated on an 8% acrylamide gel, blotted to nitrocellulose, and probed with a rat meprin β antibody. After conjugation to an HRP-linked secondary antibody, meprin bands were visualized with a chemiluminescent HRP substrate. Out of the 8 potential meprin β clones (lanes 1-8), 6 clones expressed the meprin protein to varying degrees. The standard (std) is a membrane protein fraction from rat meprin β expressing 293 cells. Protein molecular weight markers are shown at the left.

FIGURE 2. Western blot analysis of the trypsin activation of the meprin α and β proteins expressed in MDA-MB-231 (lanes labelled 231) and HEK 293 (lanes labelled 293) cell lines. Media concentrates (for meprin α) and total membrane proteins (for Meprin β) were incubated with trypsin (25 ng/μl) for 30 minutes at room temperature, which will remove the prodomain if it is present. Trypsin treated proteins (+ lanes) show a shift in molecular weight compared to non-trypsin treated controls (- lanes). The change in the size of the meprin proteins after trypsin treatment is consistent with removal of the prodomain. This indicates that the meprin proteins are not activated by the MDA-MB-231 cells themselves.

MDA-MB-231 Meprin β Transfectants

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Structure of the mouse metalloprotease meprin β gene (*Mep1b*): Alternative splicing in cancer cells

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Abstract

The mouse meprin β gene encodes an integral membrane protease that is expressed in a tissue-specific manner in embryonic and adult epithelial cells, and in carcinoma cells. The meprin β mRNA in the embryo, kidney and intestinal cells is 2.5 kb, whereas the isoform in carcinoma cells (β′ mRNA) is 2.7 kb. The work herein was initiated to explore the molecular mechanism responsible for the different isoforms. Overlapping fragments containing the *Mep1b* gene were obtained from a yeast artificial chromosome clone using polymerase chain reactions. The gene spans approximately 40 kb and consists of 18 exons and 17 introns. The first three exons are unique to the 5′ end of β′ mRNA; the next two exons correspond to the 5′ end of β mRNA. The rest of the exons (13 total) encode the regions common to both β and β′ messages. In conjunction with the cDNA sequences, the gene structure establishes that alternative splicing of 5′ exons is responsible for the generation of the mRNA isoforms. The DNA regions between β′- and β-specific exons and upstream of the first β′ exon have been completely sequenced to identify potential regulatory elements for β and β′ transcription. There is significant homology between the two regions, indicating that a duplication event occurred during evolution of the *Mep1b* gene. Potential promoter elements and transcription factor-binding sites were identified from comparisons to sequences in the databanks. This is the first gene structure that has been completed for meprin subunits from all species. The work elucidates molecular mechanisms that regulate differential expression of the *Mep1b* gene.

Keywords: Chromosome 18; Differential expression; Exon/intron organization; Protease

1. Introduction

Meprins are members of the 'astacin family' and 'metzincin superfamily' of metalloendopeptidases (Bond and Beynon, 1995; Stöcker et al., 1995). All the members of the superfamily contain zinc firmly bound at the catalytic center, and act extracellularly. Meprins are highly glycosylated, disulfide-linked oligomeric proteases composed of one or two evolutionarily related subunits, α and β. The homo- or heterooligomers that contain meprin α subunits are referred to as meprin A (EC 3.4.24.18); meprin B (EC 3.4.24.63) is a homooligomer of β subunits. The subunits are expressed embryonically and after birth in a strain-, tissue- and cell-specific manner; expression is particularly abundant in proximal tubule cells of mammalian kidney and in intestinal epithelial cells (Bond and Beynon, 1995).

Meprin subunits are also expressed in a number of mouse and human cancer cells, and this is of interest because extracellular proteases are capable of influencing the course of growth and metastases (Dietrich et al., 1996; Lottaz et al., 1999; Matters and Bond, 1999b). The 5′ end of the human meprin β gene (*MEP1B*) has recently been analyzed, and a PEA3 element was identified as being responsible for *MEP1B* expression in

Abbreviations: AM, after MATH; bp, base pairs; C, cytoplasmic; EGF, epidermal growth factor-like; kb, kilobases; LPH, lactase phlorizin hydrolase; MAM, meprin, A-5 protein receptor protein-tyrosine phosphatase μ; MATH, meprin and TRAF homology; nt, nucleotide; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SI, sucrase–isomaltase; TM, transmembrane; YAC, yeast artificial chromosome.

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cancer cells (Matters and Bond, 1999b). Meprins are capable of degrading bioactive peptides such as bradykinin, gastrin, substance P, neurotensin, and TGF-α, peptide hormones such as luliberin, parathyroid hormone, α-melanocyte-stimulating hormone, and glucagon, and proteins such as protein kinases, type IV collagen, laminin, fibronectin, and gelatin (Bond and Beynon, 1995; Chestukhin et al., 1997).

Meprins contain multidomain subunits. The deduced amino acid sequences of the α or β subunits from mouse, rat, and human are 75–90% identical, and the α and β subunits from the same species are approximately 50% identical. The predicted domain structure of the β subunit (Fig. 1A) consists of the following: S (N-terminal signal peptide), P (prosequence), Protease domain (catalytic, astacin-like), MAM (meprin, A-5 protein, receptor protein-tyrosine phosphatase μ), MATH (meprin and TRAF homology), AM (after MATH), EGF (epidermal growth factor-like), TM (transmembrane) and C (cytoplasmic). The domain structure of the α subunit is similar to the β subunit except that an additional I (inserted) domain is present in α between the AM and EGF domains. The I domain is essential for the C-terminal proteolytic cleavage of the α subunit in the endoplasmic reticulum, leading to the secretion of the α subunit if not associated with the β subunit at the cell surface (Marchand et al., 1995). The mouse meprin β subunit remains membrane-bound during biosynthesis, and the mature subunit is localized to the plasma membrane. The MAM, MATH, and AM domains of meprin subunits are essential for efficient transport of the protein to the cell surface and/or correct folding to generate enzymatically active proteases (Tsukuba and Bond, 1998).

The gene encoding the meprin β subunit exists as a single copy on chromosome 18 of the mouse and human genomes (Bond et al., 1995). When two mRNA isoforms were discovered, the 2.5 kb mRNA (β) in the embryo and in kidney and intestinal cells and a 2.7 kb mRNA (β′) in mouse carcinoma cells, it was proposed that alternative splicing is responsible for the generation of the forms in both mouse and human cells (Dietrich et al., 1996). However, recent work indicated that alternative splicing was not involved in expression of the human gene (Matters and Bond, 1999a). The work herein was initiated to determine the exon–intron organ-

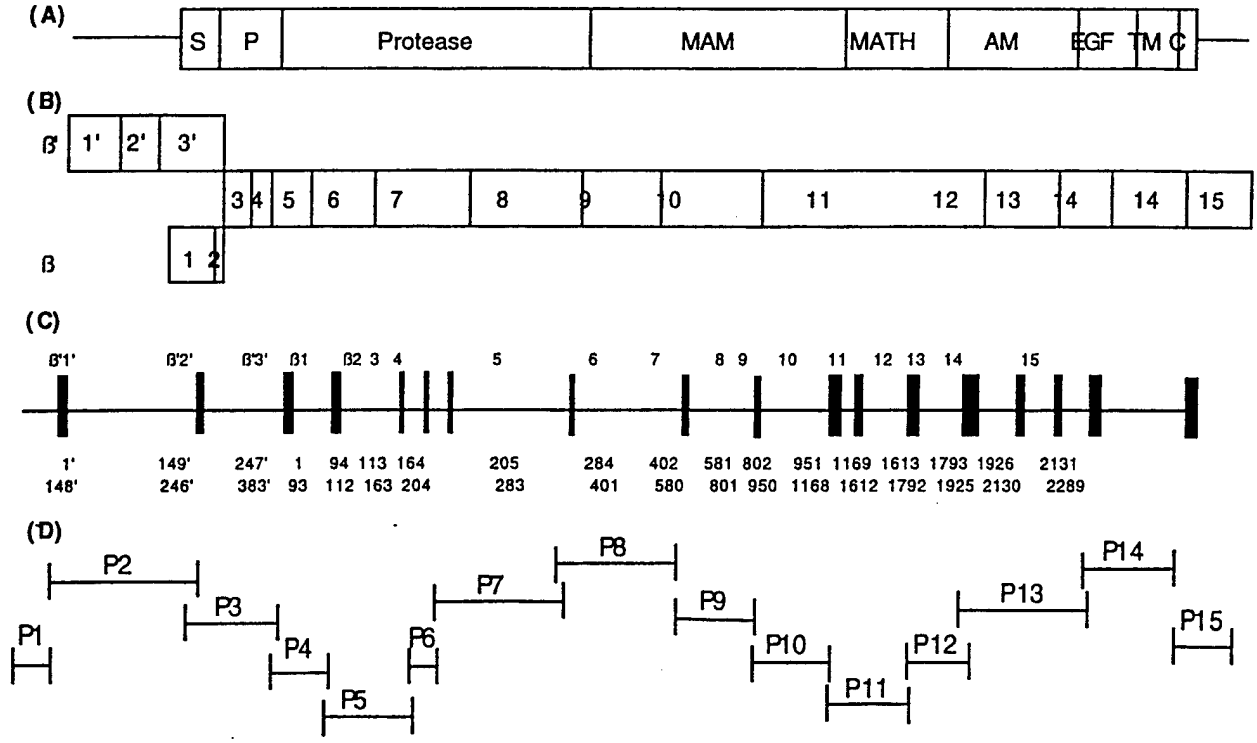

Fig. 1. Structure of the mouse meprin β subunit. (A) Protein domain structure based on the deduced amino acid sequence. The predicted functions for the domains are: S, signal peptide; P, prosequence; Protease, catalytic; MAM (meprin, A-5 protein, receptor protein-tyrosine phosphatase μ), MATH (meprin and TRAF homology) and AM (after MATH), adhesion and interaction; EGF, epidermal growth factor-like; TM, transmembrane; and C, cytosolic. (B) Exons of mRNA isoforms (β and β′). Exons 1′–3′ are unique to β′ and exons 1 and 2 are unique to β. Exons 3–15 are common for both isoforms. (A) and (B) are drawn to the same scale. (C) Exon and intron organization of the gene. Exon numbers are indicated above the exons (black bars). The numbers below exons indicate the beginning and end of the exons that correspond to the cDNA sequences (Gorbea et al., 1993; Dietrich et al., 1996). The introns and the 5′ end are represented by horizontal lines. (D) Overlapping PCR clones. Clones P1, P12, and P15 were obtained directly from the genomic DNA. All other clones were derived from YAC M63G10. (C) and (D) are drawn to the same scale.

ization for the mouse meprin β subunit gene (*Mep1b*), thereby providing the structural basis for differential expression of the two mRNA isoforms in the mouse cells.

2. Materials and methods

2.1. YAC cloning

Two mouse yeast artificial chromosome (YAC) libraries (Larin et al., 1991; Chartier et al., 1992) were kindly provided by Ellen Brundage and Craig Chinault from the Cloning Core of the Human Genome Center at Baylor College of Medicine. Multistep polymerase chain reaction (PCR) screening of the YAC libraries was performed with two gene-specific primers, AGACTCTGGCTTCTTCATGCATTTC (nt 951–975) and CACCACCCTGCTGGCCTGTAGTG (nt 1141–1119) (nucleotide numbering the same as that reported by Gorbea et al., 1993). All of the PCR reactions were carried out with the GeneAmp PCR system 9600 (Perkin-Elmer). The library was amplified in 20 μl reactions containing 10 μl of DNA and 10 μl of mix (final concentrations: 1 μM each primer, 0.2 mM dNTP, 1 × PCR buffer, and 0.05 unit of AmpliTaq polymerase). The cycling parameters were 40 cycles of 94°C/1 min, 55°C/1 min, and 72°C/1 min after the initial denaturation (94°C/5 min). Subsequent appropriate fractions were amplified as above. Positive cells (2 μl, double row/double column) were amplified with 18 μl of mix containing components that resulted in the same final concentrations as those used in primary and secondary screening. The final screening was performed in 20 μl of mix with one half of the isolated colony from plates. The PCR products from primary, secondary and final screening were detected by agarose gel electrophoresis followed by ethidium bromide staining. The PCR products from the tertiary screening were detected by Southern analysis.

2.2. PCR cloning

Exon-specific primers were synthesized based on the cDNA sequences of the mouse meprin β isoforms and the partial gene structure of the mouse meprin α subunit (Gorbea et al., 1993; Dietrich et al., 1996; Jiang and Flannery, 1997). Additional gene-specific primers were synthesized, based on newly determined intron sequences (Table 1). Gene-specific primers were used as primers (1 μM each) in PCR with either the genomic DNA or the YAC DNA (4 ng/μl) as template. The following cycling parameters, 94°C/2.5 min, 30 cycles of 94°C/1 min–57.5°C/1.5 min–72°C/1.5 min, and 72°C/5 min, were used to clone the internal regions using two gene-specific primers. In order to obtain the 5′ and 3′ fragments, a PromoterFinder DNA Walking kit (Clontech) was used. In the primary PCR, five genomic libraries in the kit were amplified with gene-specific primers and AP1 through seven cycles of 94°C/2 s and 70°C/3 min, 37 cycles of 94°C/2 s and 65°C/3 min, and 65°C/4 min. The resulting products were diluted 50-fold and amplified with nested gene-specific primers and AP2 in the secondary PCR using the same cycling parameters as those used in the primary PCR except that 20 instead of 37 cycles were performed. The PCR products were separated by agarose gel electrophoresis and isolated using the GeneClean procedure (Bio101). The fragments were cloned into a plasmid vector (pCRII or pCR2.1) using the TA cloning kit (Invitrogen). The resulting clones (P1–P15) were cleaved by restriction enzymes to determine the sizes of the fragments and partially sequenced.

2.3. Genomic Southern

Genomic DNA (10 μg) from C57BL/6 mice was digested overnight with *Eco*RI, *Hin*dIII or *Mun*I (Life Technologies). After separation on a 0.6% agarose gel, the DNA was transferred to a Nytran Plus nylon membrane (Schleicher and Schuell) and UV-cross-linked. The membranes were probed overnight with PCR-labeled DNA from either mouse *Mep1b* exon β′3′ or *Mep1b* exon β1 at 42°C in 5× SSPE/50% formamide/5 × Denhardts/1% SDS/100 μg/ml of herring sperm DNA. Blots were washed with 2× SSPE/0.1% SDS at 50°C, 0.2× SSPE/0.1% SDS at 55°C, and 0.1 × SSPE/0.1% SDS at 55°C, and exposed to Kodak X-OMAT Blue film for 3–7 days.

2.4. Sequencing

The ends of the clones were sequenced directly using the primers (SP6, M13 reverse primer, and T7) for the vector (pCRII or pCR2.1). Internal regions were sequenced by either generating deletion clones with restriction enzymes or using gene-specific primers synthesized based on the determined sequences. Both manual and automated sequencing were performed on the double-stranded DNA. Manual sequencing was performed with Sequenase 2.0 (Amersham). Automated sequencing was performed with AmpliTaq DNA polymerase, FS, using ABI Prism automated DNA sequencer (Perkin-Elmer) in the Molecular Genetics Core Facility of the Penn State's College of Medicine. Clones P1 and P4 were completely sequenced. Other clones were partially sequenced, and exon/intron boundaries were sequenced on both strands.

2.5. Sequence analyses

The determined genomic sequences were compared to the cDNA sequences of β and β′ mRNA to identify

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Table 1
Oligonucleotide sequences of the primers[a]

Clone	Size (kb)	Sense Primer	Position in cDNA	Antisense Primer	Position in cDNA
P1	0.6	GTAATACGACTCACTATAGGGC(AP1)	Not applicable	GAGCTCCAAACCAGCAGTGCTTCTTCC	96′–70′
		ACTATAGGGCACGCGTGGT(AP2)	Not applicable	CAGGCGTTGGTTCGCCAAATTGTTG	50′–25′
P2	5.0	CTGGCTGGTCTCAACAAT	14′–31′	CGGGTTGAGAAATAATGG	Intron after β2′
P3	3.1	CCTTCTCCCCTTTTCTTT	224′–241′	ATCATGTACATCCCGTCC	372′–355′
P4	1.6	AGCAGAAGCAGACACAGC	302′–319′	GGAGAAATGTGGCAAAAA	80–73
P5	3.9	ATGGATGCCCGGCATCAGCC	31–50	TGTCTTGGTCAATTCCTCCAT	145–125
P6	0.8	CAAAGACATAGATGGAGG	114–131	GAGTTTGATGTCTCCCTC	201–184
P7	4.0	GGTCTGGACCTTTTTGAG	169–186	GGCCATCTCTTGTGGTCT	248–231
P8	3.8	GCCACATACCATTCCATA	246–253	GCCCTTGAACACTGAGAT	396–379
P9	2.1	CGTGCATTGACTTCAAGCCTTGGT	338–361	TCCAATGGACAACTCCTGCTTC	456–435
P10	2.5	GGTCTTCAGTGGGAAACATTCATG	407–430	ATGGTAGACTCTGTCCCG	704–687
P11	2.5	GCACTACAGTAAAACCGCTTTCCA	660–683	CGGGTGTAAATGTTCCAGTTG	1111–1093
P12	2.2	AGACTCTGGCTTCTTCATGCATTTC	951–975	CCAGATATGGTGAGGACACCTTGT	1329–1306
P13	3.9	ACAAGGTGTCCTCACCATATC	1306–1326	CTCACACCTTTTGCCCATGTA	1971–1951
P14	2.8	CTGCAGGAGAAGACTGGT	1931–1948	TTCTTCTATGATGGAAGGTCTCTTT	2199–2175
P15	1.5	CAGCTGGGAGGGATGTCAGGAATCTG	Intron after β14	GTAATACGACTCACTATAGGGC(AP1)	Not applicable
		CACGCCAGCAGGTGAAATGAAAAGAG	2156–2180	ACTATAGGGCACGCGTGGT(AP2)	Not applicable

[a] Positions of the primers (nucleotide number) are based on the published cDNA sequences (Gorbea et al., 1993; Dietrich et al., 1996). AP1 and AP2 are adaptor primers for the genomic walking. Clones (P1–P15) are numbered from the 5′ end of the gene. P1, P12, and P15 were generated from the genomic DNA; other clones were generated from the YAC DNA. Sizes of the clones (kilobases) were estimated from the restriction analysis of the plasmid DNA.

boundaries between exons and introns. Multiple sequences were aligned using Clustal W (Thompson et al., 1994). Potential promoter regions were predicted using the NNPP (Promoter Prediction by Neural Network) method (http://www-hgc.lbl.gov/projects/promoter.html). The basis for this method is a time-delayed neural network that consists mainly of two feature layers, one for recognizing the TATA box and one for recognizing the 'initiator', which is the region spanning the transcription start site. The resulting prediction is a 50 bp region with the transcription start site at base 41 with the positional accuracy ±3 bp. Potential transcription factor-binding sites were predicted from the TRANSFAC database (version 3.5) using MatInspector (Quandt et al., 1995).

3. Results and discussion

3.1. Cloning of the Mep1b gene

To begin the analysis of the mouse meprin β gene structure, three clones (M324G12, M66E3, and M63G10) were isolated from 53 000 clones by multistep PCR screening of two YAC libraries. Southern analysis of the three clones using the full-length mouse β cDNA revealed that they all contained the mouse *Mep1b* gene (data not shown). Twelve fragments, sizes ranging from 0.8 to 5 kb, were amplified by PCR from YAC M63G10. The resulting plasmid clones were designated as P2-P11, P13, and P14 (Table 1). Clone P12 was amplified directly from the genomic DNA as described previously (Dietrich et al., 1996). Clones P1 and P15 were generated by genomic walking. Sequencing showed that all these clones were overlapping and constituted a continuous genomic fragment of approximately 40 kb.

3.2. Exon/intron organization of the Mep1b gene

The exon and intron organization of the *Mep1b* gene is presented with the domain structure of meprin β subunit protein and two mRNA isoforms (β and β′) (Fig. 1). There is no correspondence between exons and protein domains (Fig. 1A and B); some domains (e.g. the protease domain) are encoded by several exons, and others (e.g. the MATH domain) are contained within one exon. The exon/intron boundaries of the *Mep1b* gene corresponding to the protease domain are conserved in the *Mep1a* gene encoding the mouse meprin α subunit (Jiang and Flannery, 1997). Fifteen and 16 exons constitute the β and β′ mRNA, respectively (Fig. 1B). The last 13 exons are common to both β and β′ mRNA. The β′-specific exons (β′1′ to β′3′) precede the β-specific exons (β1 and β2). This type of organization provides evidence for the proposition that production of the β′ mRNA isoform involves alternative splicing of the β-specific exons from the mRNA precursor.

Exon/intron junctions of the mouse meprin β subunit gene are presented in Table 2. All the introns match the 'gt–ag' consensus sequence (Horwitz and Krainer, 1994). The sizes of the introns range from 0.5 to 4.9 kb,

Table 2
Exon/intron junctions of the mouse meprin β subunit gene[a]

[a] Nucleotides of exons and introns are in upper- and lower-case letters, respectively. Corresponding amino acids are indicated as one-letter codes below the first nucleotides of the respective codons. The sizes of introns (kb) are indicated. Phases of introns refer to position of an intron relative to codon. Phases 0, I, and II indicate the presence of an intron between two codons, between the first two nucleotides of a codon, and between the last two nucleotides of a codon, respectively (Patthy, 1987). The size of 4.9 kb includes exons β1 and β2.

Exon-Exon	Splice Donor	Size (kb)	Splice Acceptor	Phase
ß'1'-ß'2'	GAGAAG gttggtacgg G V	4.7	cctcttcaag ATACTG K D	
ß'2'-ß'3'	TATAAG gtgtgttccc	3.0	gtgacaccag GTTTCC	
ß'3'-3	TTGGAG gtaagctaaa G V	4.9*	gtaatttcag TCAAAG K D	I
ß1-ß2	GGTTTG gtaagaaaat G L	2.8	tgtttcccag CCAGCT P A	0
ß2-3	AGTTTG gtaagtctat F V	0.5	gtaatttcag TCAAAG K D	I
3-4	ACCAAG gtttgtggct Q G	0.7	cttctcttag GTTTGG L G	I
4-5	CTCGAG gtgagttgca L E	4.0	tttttgacag GCAAAT A N	0
5-6	GCTTGG gttagtacac L E	3.7	cttctgtcag AAATGA M N	I
6-7	CAGTGG gtaagttcga S G	2.0	tctgtatcag GTGCTG C W	II
7-8	AGCCAG gtatgtttct P G	2.2	atatattcag GCAAGG K E	I
8-9	ACTGCA gtatgtgatg C T	0.5	gcttttgtag CTTCTT S S	I
9-10	GCAAAG gtaacaggtt K D	1.3	ctctgagcag ACTCTG S G	I
10-11	TAAAAG gtacagtacc K E	1.8	ctttctgcag AGGTAC V P	I
11-12	CCAGTG gttcgtggct S D	1.3	tgcttttcag ATAATG N G	I
12-13	TTGAAG gtatcgaaat E D	1.1	ccctctgcag ACATAT I S	I
13-14	GTGCAA gtgaggactc C K	1.0	cttcctgtag GTGTCC C P	II
14-15	GAAAAC gtaagttgag E N	2.6	ctcctttcag CAACAT Q H	0

as compared to the sizes of the exons from 19 (β2) to 444 (exon 11) bp (Fig. 1C). Less than 6% of total DNA (40 kb) are exonic sequences. The majority of the introns (11 out of 15 that are present in the coding region) are phase I introns, and there are three phase 0 and two phase II introns, respectively (Patthy, 1987).

3.3. Mouse genomic Southern

Because the genomic structure of the *Mep1b* gene was derived from PCR-amplified YAC clones, a genomic Southern analysis was used to confirm the location of the β′ exons in mouse DNA. Based on the YAC sequences, a partial restriction map of the first three β′ exons and the first five β exons was made and used to select enzymes for cleaving the genomic DNA (Fig. 2A). As predicted from the map, the same *Hin*dIII and *Mun*I fragments were detected by the β′3′ exon probe and by the β1 exon probe (Fig. 2B). This indicates that the two exons are adjacent to each other in the genomic DNA. The YAC clone would also predict that the β′3′ exon probe and the β1 exon probe would hybridize to different *Eco*R I fragments. The different *Eco*RI fragments detected by the β′3′ exon probe and the β1 exon probe indicate the the β′3′ exon is upstream of the β1 exon. Taken together, the genomic Southern results confirm that the β′ exons are not artifacts of YAC cloning or PCR amplification and exist in the mouse genome in the same location as in the YAC cloned DNA.

3.4. Potential promoter elements for the β isoform

The 1.6 kb sequence including the region immediately upstream of the first β-specific exon was determined

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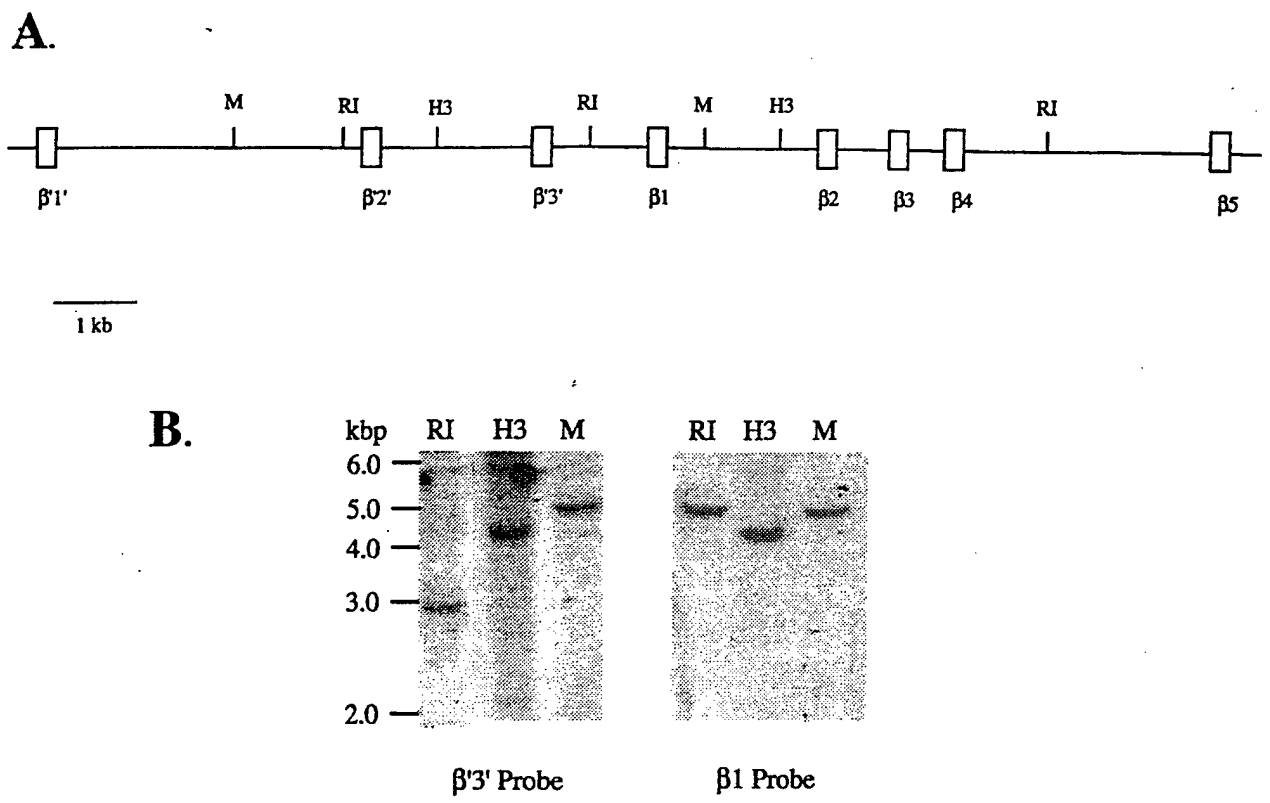

Fig. 2. Mouse genomic Southern. (A) Schematic of the intron–exon organization of the 5′ end of the mouse *Meplb* gene. Boxes represent the exons, and restriction sites are indicated by the vertical lines. Abbreviations for the restriction sites: R1 = *Eco*RI, M = *Mun*I, H3 = *Hin*dIII. (B) Genomic Southern blots of C57BL/6 mouse DNA digested with *Eco*RI, *Hin*dIII or *Mun*I. Molecular-weight markers (kbp) are listed at the left. The membrane on the left was hybridized with a probe to *Meplb* exon β'3', while the membrane on the right was hybridized with a probe to *Meplb* exon β1.

from clone P4 because this region may contain potential promoter and other regulatory elements for transcription of meprin β mRNA in kidney and intestinal cells (Fig. 3). Three potential promoters were predicted for β using the NNPP method with the score cut off of 0.8. They are located in the regions of nt 502–551, 632–681, and 1492–1541 (indicated by dotted lines) with the score of 0.92, 0.99, and 0.95, respectively. The transcription start site of the last promoter region (adenosine, A, double-underlined) coincides with the 5′ end of cloned β cDNA obtained by the 5′ RACE procedure (rapid amplification of cDNA ends) (Gorbea et al., 1993), consistent with the proposition that this is the site for initiation of transcription. Therefore, the nucleotide A is designated as the first base in the β1 exon.

Many potential transcription factor-binding sites were predicted in the 1.5 kb sequence upstream of the β1 exon from the TRANSFAC database (version 3.5) using MatInspector (Quandt et al., 1995). Fig. 3 indicates the sites that matched the consensus sequences with 100% core similarity and at least 95% matrix similarity. Transcription factors HFH-3, CREB and GATA-1 are strong candidates for regulating kidney and intestinal expression of the *Meplb* gene based on the following observations. HFH-3 is a winged helix transcriptional activator expressed in the distal tubules of embryonic and adult mouse kidney, and the HFH3 site is found in several kidney-specific genes such as Na/K-ATPase and E-cadherin and transcription factors such as HNF-1 and HNF-4 known to regulate gene expression in kidney and intestine (Traber and Silberg, 1996; Overdier et al., 1997). CREB (cAMP response element-binding protein) interacts with an intestinal homeodomain transcription factor Cdx2 and enhances Cdx2-dependent transcriptional activity (Lorentz et al., 1999). Several members of GATA-binding proteins are implicated in gene regulation in intestine (Laverriere et al., 1994).

There are also factors upstream of the β1 exon that may be important for developmental regulation of the *Meplb* gene. For example, the murine S8 homeobox gene is expressed in a mesenchyme-specific pattern in embryos and in regions involved in epithelio-mesenchymal interactions (de Jong et al., 1993). Brn-2 is a developmental regulator containing a POU domain (Li et al., 1993). Other potential transcription factors recognizing the sites indicated in Fig. 3 have been shown to regulate a variety of genes. For examples, Sox-5 is a novel murine gene related to SRY, the testis-determining gene, and highly expressed during spermatogenesis

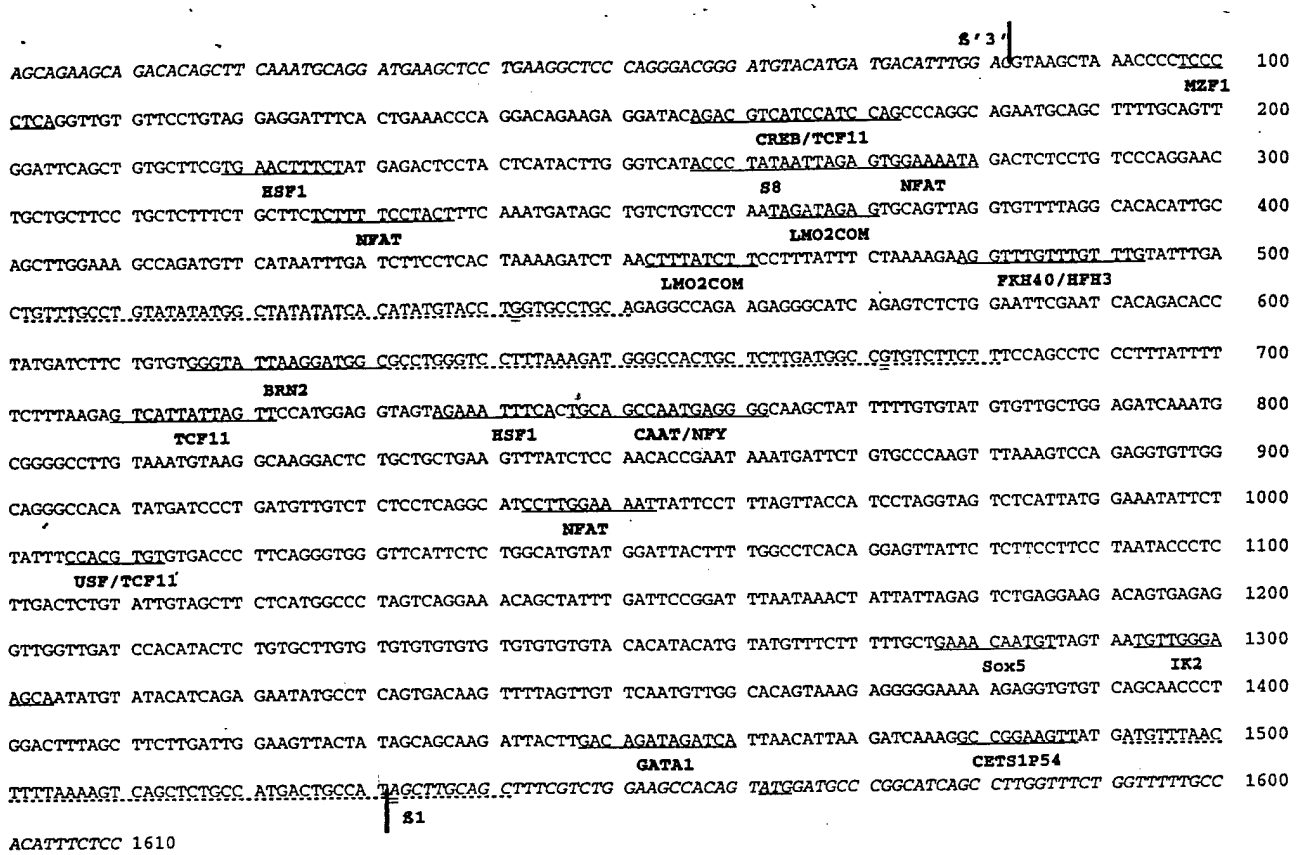

Fig. 3. Mouse *Mep1b* sequence between exons β'3' and β1. The complete sequence of clone P4 (Fig. 1D) is numbered on the right. The sequences of the primers used to generate this clone correspond to the first and last 18 nucleotides, respectively (Table 1). The partial sequences of exons β'3' (1–82) and β1 (1532–1610) are italicized, and the translation start codon for the β protein is underlined. The end of exon β'3' and the start of exon β1 are marked by vertical bars. Potential promoter regions are indicated by dotted lines with transcription start sites double-underlined. Potential transcription factor-binding sites are indicated. The sequence has been deposited in GenBank with Accession No. AF160982.

(Denny et al., 1992). MZF1 and Lmo2 play a role in hematopoiesis (e.g. Perrotti et al., 1995). IK-2 is one of the zinc finger DNA-binding proteins encoded by the lymphocyte-restricted Ikaros gene, the master regulator during lymphocyte development (Molnár and Georgopoulos, 1994). In contrast, TCF11 is more widely expressed, and HSF-1 is a member of the heat-shock transcription factors known to function in the cellular stress response (e.g. Johnsen et al., 1998). The oncoprotein c-Ets-1(p54) that binds the CETS1P54 site is known to activate the promoter of a matrix metalloproteinase stromelysin (Wasylyk et al., 1991). The NFY-binding protein, NF-Y, is the major CCAAT box recognizing protein that may serve different roles in TATA-containing and TATA-less promoters (Mantovani, 1998).

3.5. Comparison of the 5' upstream sequences of β and β' and potential promoter elements for the β' form

The 0.5 kb sequence containing the region immediately upstream of the first β'-specific exon was determined from clone P1. Fig. 4 indicates potential transcription factor-binding sites in the β' upstream region that matched the consensus sequences in the TRANSFAC database (version 3.5) with 100% core similarity and at least 95% matrix similarity using MatInspector (Quandt et al., 1995). Four of the sites, HSF1, GATA1, S8 and IK2, are also present in the β upstream region, but their positions are not conserved. The GATA1 site is also present in the promoter region of the human gene encoding matrix metalloproteinase matrilysin (Wilson and Matrisian, 1998). Two sites, TATA and CDPCR3HD, are unique to the β' upstream region. The CDPCR3HD site is recognized by a Cut-like protein that belongs to a distinct class of homeodomain proteins with multiple DNA-binding domains and acts as a negative regulator of gene expression (Harada et al., 1995).

The sequence consisting of the upstream region and the β'-specific exons is 42% identical to the β sequence in more than 900 bp (Fig. 4). The degree of homology between the two different regions of the *Mep1b* gene indicates that a duplication event occurred during the evolution of this gene. Following the duplication event,

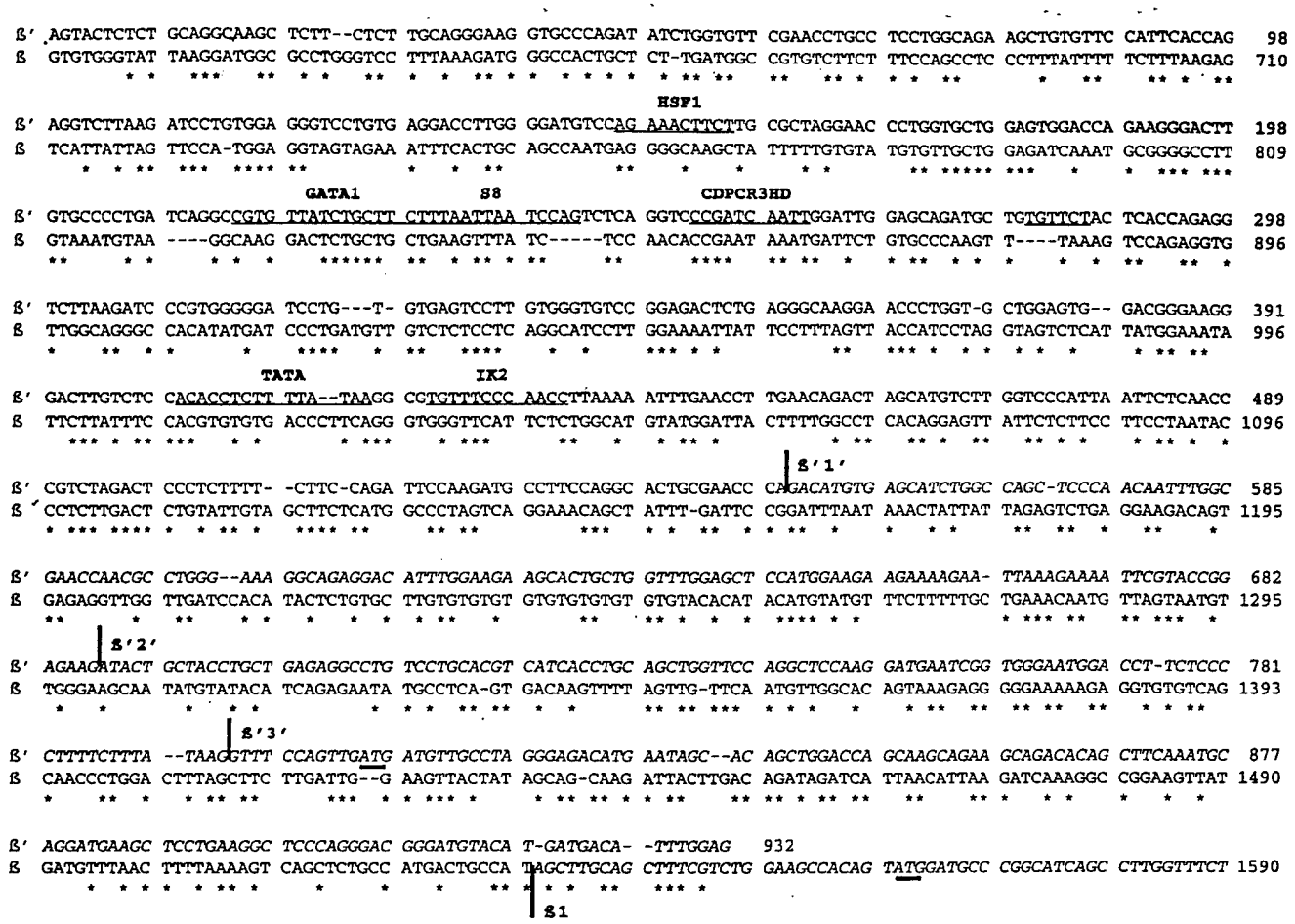

Fig. 4. Comparison of *Mep1b* genomic sequences upstream of β′ and β isoforms. The sequences were aligned using Clustal W (Thompson et al., 1994). The β sequence is numbered the same as in Fig. 3. For the β′ sequence, the genomic part (1–599) is derived from clone P1, and the cDNA part (600–932) is derived from exon β′1′–β′3′ corresponding with the published sequence of nucleotides 51–383 (Dietrich et al., 1996). Identical nucleotides between the two sequences are indicated below the β sequence by asterisks. Potential transcription factor-binding sites for the β′ sequence are underlined and indicated above the β′ sequence. The sequences found in the cDNA are italicized, and the translation start codons are indicated by short horizontal bars. The start of each exon is marked by vertical bars. The sequence of clone P1 has been deposited in GenBank with Accession No. AF160983.

either new sequences (introns) were inserted between the β′-specific regions, or the corresponding sequences were eliminated from the 5′ upstream region of β.

3.6. Comparison of the regulation of meprin β expression in mouse and human cells

To compare the potential tissue-specific elements identified for the mouse and human meprin β genes, the sequences from the two species upstream the β mRNA isoforms were aligned (Fig. 5). The low homology is reflected by many and large gaps introduced for the optimal alignment of the two sequences. Furthermore, the putative intestine-specific elements indicated for the human gene, SI/cdx2 and LPH/cdx2, are not conserved in the mouse gene, and the putative kidney-specific elements identified for the mouse gene, HFH3, CREB and GATA1 (Fig. 3), are not found in the human gene. However, several short motifs (six to 10 nucleotides) are conserved in the mouse and human genes (Fig. 5). It is possible that these motifs represent novel intestine- and kidney-specific elements for expression of the meprin β genes in the two species.

The factors responsible for expression the meprin β′ mRNA isoforms in human and mouse cancer cells may be different. For example, an AP1/PEA3 site implicated in expression of human β′ (Matters and Bond, 1999b) is only partially conserved in the mouse gene (Fig. 5). No element known to affect gene expression in cancer cells was identified for the mouse gene in the 500 bp sequence upstream of the β′ mRNA isoform, indicating either that elements are located further upstream or that there are novel elements for expression of the mouse gene in cancer cells.

The results herein indicate that the mechanisms responsible for expression of the meprin β gene in cancer cells from mouse (*Mep1b*) and human (*MEP1B*) are different (Fig. 6). Alternative splicing is involved in

```
                                  1/2 ERE
MEP1B  CGACCTCAGGTGGTCCACCCACCT-TGACCTCCCAAACTGCTGGGATTACACATGTGAGCCACCATGGCCAAAAATTAGTATTCAAACAATATTATCCTG    99
Mep1b  AGAGGT--GTTGGCAGGGCCACATATGA--TCCCTGA-TGTTGTCTCTCCTCAGG----CATCCTTGG---AAAAT---TATTC---CTTTAGTTAC---  968
       ** * * ***     **** * ***  ****  * ** **    * * ** *   *  ** ***   *****  *****  * ** * *
                                                                                          PEA3
          C/EBP δ
MEP1B  CATCTTAAGTAAGTTGAATATGAAGCCATGGGAACTGAGAGAACAGGTTGTTTCTAAAGAGAAATTATCCCAAGAAAAGAAGGAAAAAGTGAGAAAGAAT    199
Mep1b  CATCCTAGGTAGTCTCATTATGGA----------------AATA------TTCTTA--------TTTCC----------------ACGTGTGTGACCCT   1021
       **** ** ***   * * **** *                  ** *      **** *       * ***                  * *** * *  *
                          SI/cdx2          1/2 ERE          1/2 ERE/AP1
MEP1B  TAAGAGATGGATTGGGATCATTATTTTATTAGAAAATCAGTGACCATTCCTACTTTTATGACCCATGTCCCACCAGTGTTTG-CTTGATGGGAATTTTTA    298
Mep1b  TCAGGG------TGGGTTCATTCTCT----GGCATGTATG-GAT------TACTTT--TGGCC----TCACAGGAGTTATTCTCTTCCT------TCCTA   1092
       * * *      **** ***** * *     * *  *  * **        ******  ** **    ** **  ***  **  ***  *     *  **
                                                                                          SI/cdx2
MEP1B  GGGATGAGATACATTGCTATGTTACTACTCTATATGTGAATAACCGGTGTGCCTGATTTC-TAGCAC-ATTAATGGATTTTATAACTCAATAAATTATAG    396
Mep1b  --------ATACCCT-CT-TG-----ACTCTGTAT------------TGTAGCT--TCTCATGGCCCTAGTCA-GGAA---ACAGCT-ATTTGATTC--C   1156
              ****  * ** **      ***** ***           ***  **  * ** * ** * * * * ***    * * ** * *  ***
                   SI/cdx2          SI/cdx2
MEP1B  GCACTCAATAATTT-TTATTAAATGGGTGAATGATAAAAAGCAGTGGACATAACTGGATATATTAAAGCAGCATGGGGCTATATGAGAGGAGGAGTTTCA    495
Mep1b  GGATTTAATAAACTATTATTAGA--GTC---TGAGGAAGA-CAGTG---AGAGGTTGGTTGATCCACATACTCTGTGCTTGTGTGTGTGTGTGTGT--   1245
       * * * *****  * ******  * *      ***  ** * *****  * *  * * *  **  *   *  ** *  * * ** * *   *  * *
                                                nkx2.5  LPH/cdx2
MEP1B  TAAGATCAAGATTAAAAATTAATTTGATATATATATTTTTTGCTTAAGTGGTGTTTACTAGTCTAAGGAC-CAGTACACATACATTAGAGAATGTAT-TC    593
Mep1b  ---------GTGTACACATACAT---GTATGT-TTCTTTTTGCTGAAACAATGTT-AGTAATGTTGGGAAGCAATATGTATACATCAGAGAATATGCCTC   1331
             * ** * **  ***** * * * *  ******** **     **** * ** * *  ***  ** **   ******  *******  *  **
                        nkx2.5    AP1/PEA3                OSE2                          TATA
MEP1B  ATTAAGAAGTCTCAGCCAGGCACTTAATTGTTGACACAGGAAAAAAAAAAGCCAACAAACCAACTCCGCAGAGCTGTGTTAAAAATACTGGACTTTCATC    693
Mep1b  AGTGACAAGTTTTAGTTG-----TTCAATGTTGGCACAGTAAAGAG----GGGGAAAAA-----------GAGGTGTGTCAGCAACCCTGGACTTTAGCT   1411
       * * * **** * **        ** * ***** ***** *** *     *   * ***          *** ***** *  **  *********
          δ'                                                                    TATA
MEP1B  CCTTGATTGGAGGTATTATTACAGCATCAAGCTGACCTGCAATATGGATCATTGA------GACCAAAGGCTATAAATTATAATGTTTGCCTTTAAAGAA    787
Mep1b  TCTTGATTGGAAGT--TACTATAGCAGCAAGATTACTTGACAGATAGATCATTAACATTAAGATCAAAGGCCGGAAGTTATGATGTTTAACTTTTAA-AA   1458
       **********  **  ** ** **** **** * ** **  * ** *******  *      ** *******  ** **** ******  **** ** **
          δ
MEP1B  GTCAATTCAACCCTGAATGTCATGGTTAGCTACTTTCAACTGGAAGCTACAACATGGATTTATGGAATCTGTCTTGGTTTCTGTTCTTGGATGCTCTTCTCG    889
Mep1b  GTCAGCTCTGCCATGACTGCCATAGCTTGCAGCTTTCGTCTGGAAGCCACAGTATGGATGCCCGGCATCAGCCTTGGTTTCTGGTTTTTGCCACATTTCTCC   1600
       ****  **  ** *** ** ***  * * **  *****  ******** ***  ******    ** *** * ********** * ** *  *  *****
                 δ
```

Fig. 5. Comparison of human *MEP1B* and mouse *Mep1b* sequences upstream of the β mRNA isoforms. The potential transcription factor-binding sites identified for the *MEP1B* gene by Matters and Bond (1999a) are underlined and the factors indicated. Short sequences of six to 10 nucleotides, and the start codon for translation, conserved in the two genes are indicated by horizontal bars. The starts of different mRNA isoforms are indicated by arrows. SI, sucrase-isomaltase; LPH, lactase phlorizin hydrolase.

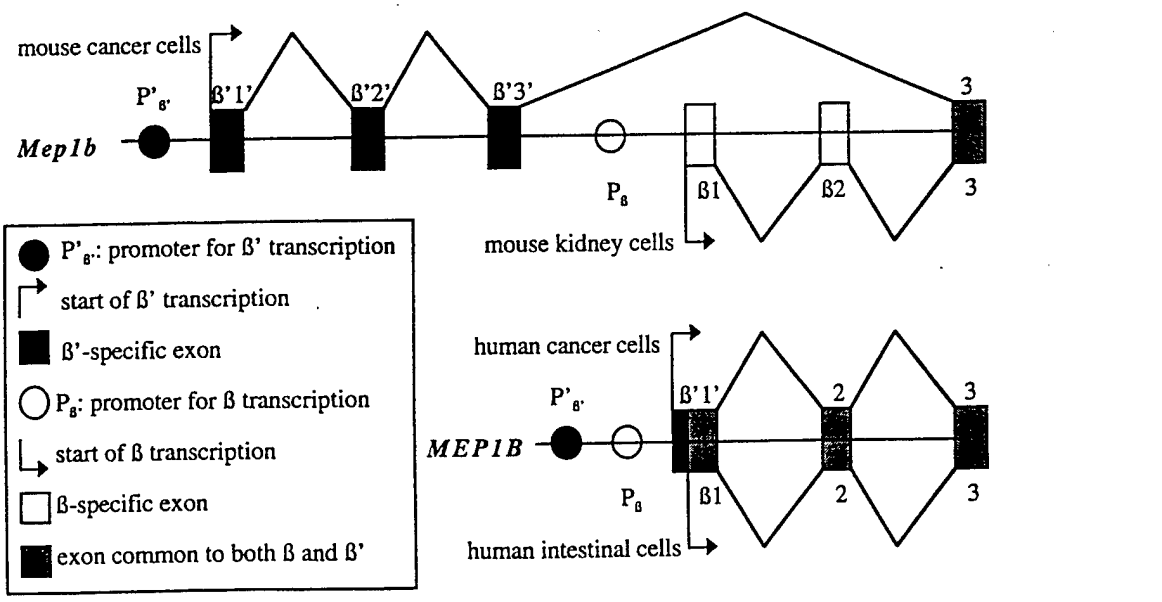

Fig. 6. Proposed mechanisms for expression of the meprin β gene in mouse and human cells. Splicing of exons β1 and β2 is involved in expression of the mouse *Mep1b* gene, not in the human *MEP1B* gene, in cancer cells.

expression of *Meplb* but not *MEP1B*. The promoter (P′β′) for directing the expression of the β′ isoform in mouse cancer cells is predicted to be several kb away from the promoter (Pβ) for the β isoform expressed in normal cells. In contrast, the two human promoters are predicted to be proximal to each other. In addition, the human proteins produced in cancer and normal cells are the same, whereas the mouse β′ protein expressed in cancer cells encodes a signal peptide and part of the prosequence that differ from the β protein produced in normal cells.

3.7. Conclusions

1. The mouse *Meplb* gene spans approximately 40 kb on chromosome 18 and consists of 18 exons and 17 introns. The first three exons compose the unique 5′ end of β′ mRNA found in mouse cancer cells; the next two exons correspond with the 5′ end of β mRNA expressed in kidney and intestinal cells. The mouse genomic Southern confirms the location of the β′ exons in the mouse DNA. The rest of the exons (13 total) encode the regions common to both β and β′ messages. In conjunction with the cDNA sequences, the *Meplb* gene structure establishes that alternative splicing of 5′ exons is responsible for the generation of the two mouse mRNA isoforms.
2. Potential promoters and transcription factor-binding sites were identified in the upstream regions of the β- and β′-specific exons. The two regions showed a significant homology, indicating that a duplication event occurred during evolution of the *Meplb* gene. However, the potential regulatory elements were not conserved, suggesting the differential regulation of the gene in normal and cancer cells.
3. A major species difference exists in expression of the meprin β and β′ isoforms between mouse and human in normal and cancer cells. Alternative splicing of 5′ exons occurs in the mouse gene expression, whereas there is no evidence of any involvement of alternative splicing in the human gene expression.

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Mouse

SUBSTRATE SPECIFICITY OF THE MOUSE KIDNEY METALLOENDOPEPTIDASES, MEPRINS A AND B. Greg P. Bertenshaw, Gail L. Matters, John Bylander and Judith S. Bond. Department of Biochemistry and Molecular Biology, Pennsylvania State University, College of Medicine, Hershey, PA 17033, USA.

Meprins A and B are zinc-dependent metalloendopeptidases of the astacin family and metzincin superfamily. They consist of evolutionarily related α and/or β subunits. A high level of expression of these secreted or membrane-bound ectoenzymes is seen in the brush border membranes of intestine and kidney proximal tubules. Meprins are capable of degrading a variety of peptides and proteins *in vitro*. Meprin A is capable of hydrolyzing gelatin, basement membrane proteins, insulin B chain and numerous peptides including bradykinin, substance P and angiotensins. Meprin B cleaves protein kinase A, insulin B chain and the peptides such as gastrin. In order to identify possible physiological substrates of meprin additional gastrointestinal peptides were examined for hydrolysis by the meprins. We found that meprins are able to degrade gastrin-releasing peptide, glucagon, secretin, cholecystokinin, peptide YY, vasoactive intestinal peptide and orcokinin. Some peptides (e.g. CCK) are susceptible to both meprin A and B, however, cleavage occurs at different peptide bonds. The data indicate that although the α and β subunits of meprins are very similar with respect to amino acid sequence (58% amino acid identity within the protease domain), they have very different substrate specificities. In this study we show that meprin A has a rather broad specificity, in contrast meprin B is much more specific with preference for aspartate or glutamate residues at the P1' site. We conclude that Meprin B is predominantly an acidic-N endopeptidase. This study focuses on a kinetic comparison of meprins A and B using established and newly identified peptide substrates. Here we report cleavage sites as well as k_{cat} and K_m values for meprins against each peptide. Furthermore we address the role of the individual subunits in the degradation of extracellular membrane proteins by meprin A. This work is fundamental to the development of specific inhibitors to the individual subunits to further delineate the function of meprins *in vivo*.

Substrate Specificity of the Mouse Kidney Metalloendopeptidases Meprins A and B

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Meprins A and B are zinc dependent metalloendopeptidases of the astacin family and metzincin superfamily. They consist of evolutionarily related α and / or β subunits. A high level of expression of these secreted or membrane-bound ectoenzymes is seen in intestine and kidney proximal tubules. There is evidence that implicates meprins in the susceptibility to renal disease; i.e., recent segregation and linkage analyses showed the meprin β gene to have major effects on the prevalence of diabetic nephropathy in Pima Indians. Meprins are capable of degrading a variety of peptides and proteins *in vitro*. The study presented here is directed towards the identification of additional physiological substrates that are susceptible to meprins. Meprin A is capable of hydrolyzing gelatin, basement membrane proteins, insulin B chain and numerous peptides including bradykinin, substance P and angiotensins. Meprin B cleaves protein kinase A, insulin B chain and the peptides gastrin, cholecystokinin and substance P among others. These data indicate that although the α and β subunits of meprins are very similar with respect to amino acid sequence (58% amino acid identity within the protease domain) they have very different substrate specificity's. This study focuses on a kinetic comparison of meprins A and B using established and newly identified substrates. This work will allow for the development of specific inhibitors to the individual subunits to further delineate the function of meprins *in vivo*.

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April 14, 2000

Gail L. Matters, Ph.D.
Department of Medicine
Division of Endocrinology

Dear Dr. Matters:

Thank you very much for applying for Cancer Center membership. I am pleased to inform you that the committee has reviewed your application and has approved it. We look forward to your future participation in all Cancer Center related activities.

Sincerely,

Andrea Manni

Andrea Manni, M.D.
Professor of Medicine